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Vibrio cholerae Enterotoxin and Its Mode of Action

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INTRODUCTION

The probability that clinical cholera results from the interaction of a toxin produced by *Vibrio cholerae* with the intestine was proposed by Koch (59) and by other early investigators of the disease. Many subsequent studies support this conclusion. The nature of this toxin and its mode of action, however, remained obscure until recent years when the development of animal models closely resembling human cholera and purification of the diarrheagenic product of *V. cholerae* have permitted major advances in our understanding of the toxin and its mechanism of action.

V. cholerae produces a number of enzymes and other products which have at times been implicated as participating in the diarrhea-producing process (9). These have been described in recent reviews (10, 11) and will not be considered here. This review will deal only with the diarrhea-producing moieties recently isolated in various degrees of purity by several investigators. These have been referred to as choleragen (34), skin permeability factor (73), vascular permeability factor (18), type-2 cholera toxin (16), cholera enterotoxin, and cholera exotoxin. Although different techniques have been employed to prepare and isolate these agents, the weight of present evidence strongly suggests that they contain the same diarrheagenic enterotoxin and that this enterotoxin is responsible for the production of the clinical cholera syndrome. In this review we have elected to employ the term cholera enterotoxin to describe this agent because its most important clinical effect is on the gut.

CHARACTERISTICS OF V. CHOLERAE ENTEROTOXIN

Recently described preparations of cholera enterotoxin (16, 17, 34, 74), whether crude or highly purified, share a number of common characteristics, an observation which strongly suggests that they contain the same enterotoxic agent. In each, the diarrheagenic activity is heat

labile at 56 C, acid labile, destroyed by Pronase but not trypsin, and antigenic, giving rise to neutralizing antibodies after parenteral injection. These enterotoxin preparations also share the properties of causing increased skin capillary permeability when injected intracutaneously and of inducing intestinal fluid loss when introduced into the small-bowel lumen.

Considerable evidence indicates that an enterotoxin with the above characteristics is responsible for the diarrhea associated with *V. cholerae* infection. Cell-free culture filtrates of *V. cholerae* isolated from humans with cholera have been shown by Dutta and Habbu (25) to induce lethal diarrhea when given by orogastric tube to infant rabbits. De and Chatterje (20) have shown that the cell-free culture supernatant fluid induces intestinal fluid accumulation when placed in the lumen of a ligated segment of adult rabbit small bowel. Sack and Carpenter (79) have demonstrated that similar culture filtrates induce fluid loss from segments of canine small bowel. Finally, Benyajati et al. (8) were able to produce diarrhea in human volunteers who were given a culture filtrate of *V. cholerae* Inaba 569B by mouth. A substance having the characteristics of cholera enterotoxin has been found in the stools of acutely ill cholera patients. Panse and Dutta (69) and Dutt (24) have shown that sterile filtrates of acute cholera stool contain a heat-labile factor which increases skin vascular permeability in guinea pigs and which is neutralized by convalescent sera from the same patients. Convalescent sera also neutralize the diarrheagenic effect (55, 71) and other biological activities attributable to cholera enterotoxin (48). Finally, parenteral immunization of dogs with cholera enterotoxin imparts significant protection against subsequent intestinal challenge with viable *V. cholerae*, the degree of protection being correlated with the level of the serum antitoxin titer (19).

Production of Cholera Enterotoxin

Enterotoxic activity in a cell-free filtrate of *V. cholerae* culture was first demonstrated by De (21) who grew the organisms in peptone-saline (5 and 0.5%, respectively). He noted that with the growth conditions employed some strains of *V. cholerae* produced enterotoxin, whereas others did not. Several workers subsequently attempted to produce cholera enterotoxin under a variety of growth conditions and confirmed the observation that many *V. cholerae* strains isolated from patients with cholera yield little or no enterotoxin when grown in vitro (30, 73). These strains are consistently diarrheagenic in vivo, and enterotoxin can frequently be identified in the fluid which accumulates in rabbit ileal loops after injection of the organisms (30). However, a number of *V. cholerae* strains do produce enterotoxin in vitro (30, 73), the most extensively studied of these being the classic strain Inaba 569B. This strain has been widely used because it produces very large amounts of enterotoxin under simpler and more widely varied growth conditions than is true for other *V. cholerae* strains (73). There is no evidence at present that the enterotoxin produced by Inaba 569B differs in any way from that produced by other *V. cholerae* strains under different conditions of growth. Enterotoxin produced by Inaba and Ogawa serotypes and by classical and El Tor biotypes have been shown to be identical by agar gel double diffusion precipitin testing (30).

Finkelstein and Lo Spalluto (34) and Evans and Richardson (27) have shown that Inaba 569B produces large amounts of enterotoxin when grown in a simple medium containing Casamino Acids, sucrose, and various salts. Alteration of the salt composition of this medium also permits enterotoxin production by several other *V. cholerae* strains (73). An even simpler medium, containing as few as four amino acids plus yeast extract and salts, has been shown to support enterotoxin production by Inaba 569B (75). Most studies have indicated that enterotoxin production requires vigorous aeration of the growth medium (16, 27), though Craig has shown that enterotoxin can also be produced by some strains in stationary cultures (18). Temperature and *p*H also influence enterotoxin production, the enterotoxin yield being greater at lower temperatures (25 to 30 C) than at higher temperatures and at a *p*H of 7.0 to 7.8 than at higher *p*H (60, 73). Several *V. cholerae* strains have been shown to produce enterotoxin only when grown at 25 to 30 C (73). It has also been demonstrated that enterotoxin produced in shaken cultures, except when produced by Inaba 569B, is unstable in the growth medium, the quantity declining rapidly when incubated for several hours after maximum content is achieved (60, 73). This loss of enterotoxin activity may be due in part to the shaking of the cultures. Kusama and Craig (60) have shown that with at least one strain of *V. cholerae* enterotoxin produced in stationary cultures is stable up to 48 hr, whereas it disappears rapidly after reaching its peak concentration in shaken cultures.

The appearance of cholera enterotoxin in cell-free supernatant fluids of *V. cholerae* culture suggests that the material may be an exotoxin. This has been confirmed by Richardson (73) who demonstrated that cholera enterotoxin appears in the culture supernant fluid well before

any visible sign of cell lysis, enterotoxic activity being demonstrable early in the log phase of growth and reaching peak levels at the transitional period between log and stationary phase growth. The origin of the enterotoxin from within the cell or cell wall and the factors which stimulate its production and release are not well understood. That the toxin is not a constant component of the bacterial cell has been demonstrated by Richardson who showed that cells lysed before entering the transitional growth phase contained no detectable enterotoxin. Enterotoxin production was related in some way to changes occurring during the log phase of growth and was proportional to the duration of the log phase. When the log phase of growth was delayed by lowering the incubation temperature, the rate of enterotoxin formation was similarly slowed. Enterotoxin release appeared to occur with a "burst" at the end of the exponential phase and onset of log phase growth (73).

Purification of Cholera Enterotoxin

Methods for obtaining a highly purified cholera enterotoxin have been described by Finkelstein and Lo Spalluto (35) and by Richardson et al. (74, 75). The former technique involves filtration of the cell-free culture supernatant fluid through membranes with graded pore sizes and chromatography on Sephadex and Agarose. The latter technique involves enterotoxin precipitation with dextran sulfate and ammonium sulfate, gel filtration, and finally chromatography on diethylaminoethyl Sephadex. Both techniques yield an enterotoxin which appears pure when examined by immunological, ultracentrifugal, immunoelectrophoretic, and disc electrophoretic techniques. Additionally, Finkelstein and Lo Spalluto (37) have described the concurrent purification of an antigenically identical but smaller molecule which has none of the biological activities of cholera enterotoxin. This they have considered a natural toxoid and have termed "choleragenoid."

Although the techniques above yield a highly purified enterotoxin, they present some difficulties in the manufacture of enterotoxin on a large scale as for vaccine production. A major simplification in technique which permits a rapid reduction of working volume and elimination of the majority of contaminating materials was introduced by Spyrides and Feeley (88) who demonstrated that the enterotoxin is selectively absorbed onto aluminum compound gels from which it can readily be eluted after centrifugation.

Properties of Purified Cholera Enterotoxin

The highly purified cholera toxins produced by Finkelstein and Lo Spalluto (37) and Richardson et al. (74) retain all the activities of cholera enterotoxin described above and in the following sections. Of particular importance is the observation that they retain skin vascular permeability activity in the same relation to diarrheagenic potency as do less-purified preparations. This is of particular importance because of the wide use of skin vascular permeability assays for measurement of cholera enterotoxin and antitoxin activity and because of the reports by Lewis and Freeman (61) and Grady and Chang (44) which suggest that a diarrheagenic toxin free of skin vascular activity can be separated from crude culture filtrates.

Purified cholera enterotoxin is 85 to 92% protein and contains no carbohydrate and less than 1% lipid (34). Estimation of its molecular size by comparing its elution volume on Sephadex G-75 with proteins of known molecular weight (MW) has suggested that it behaves like a protein with an MW of 61,000 (34). A more precise estimate of MW has recently been obtained by Lo Spalluto and Finkelstein (*unpublished data*) using equilibrium centrifugation. They now estimate MW at 90,000 and indicate that mild acid treatment of the enterotoxin yields six subunits of 15,000 MW. By similar techniques, their choleragenoid molecule has an MW of 60,000 and appears to be composed of four 15,000 MW subunits. The sedimentation coefficient of purified enterotoxin is 5.6*S*, whereas that of choleragenoid is 4.2*S* (34).

Purified cholera enterotoxin is extremely potent in terms of biological activity. As little as 0.1 ng injected intracutaneously results in delayed alteration of skin capillary permeability (J. P. Craig, *personal communication*) and 0.4 μg will cause detectable fluid accumulation in a ligated segment of rabbit ileum (30).

The purified enterotoxin has been shown to be antigenic when given parenterally to animals (71). Furthermore, the antitoxin thus stimulated protects against the biological activities of the enterotoxin including the diarrheagenic activity (31, 32). This characteristic has prompted current investigations of the possible value of antitoxic immunity in protection against natural disease. It has been shown that enterotoxin can be converted to a biologically inactive toxoid by Formalin without loss of antigenic potency (28). Since purified enterotoxin is virtually free of cell wall antigens, a toxoid prepared from it would appear well suited for evaluation of pure antitoxic immunity. Studies of dogs immunized

parenterally with purified enterotoxin have demonstrated a high level of protection against intestinal challenge with *V. cholerae* of at least 9 months' duration (N. F. Pierce, *unpublished data*).

MODE OF ACTION OF CHOLERA ENTEROTOXIN

The isolation of cholera enterotoxin in a highly purified state and the development of means of studying its effect upon isolated viable membranes and cell systems have led to recent studies which have made major contributions to our present understanding of the biochemical effects of cholera enterotoxin and the relationship of these effects to the disease produced in man by infection with *V. cholerae*. Although some gaps remain in our knowledge of the mechanism of action of cholera enterotoxin, a single hypothesis now appears to explain most of its intestinal and nonintestinal effects. The following is a review of the present knowledge of the effects of cholera enterotoxin and a discussion of the possible mechanisms by which these effects are produced.

Gastrointestinal Effects of Cholera Enterotoxin

Evidence that the diarrhea of cholera is mediated by a specific cholera enterotoxin was reviewed above. It is now clear that diarrhea results from action of the enterotoxin upon the small bowel and that this effect is produced without major alteration in mucosal morphology and with the preservation of several important active mucosal cell functions.

During natural disease, *V. cholerae* is present in large numbers throughout the entire length of the gastrointestinal tract, from mouth to anus (42). Present evidence indicates, however, that only the small bowel contributes to the production of diarrheal fluid. Perfusion of the small bowel with nonabsorbable dilution indicators was performed by Banwell et al. (5) in acute and convalescent cholera patients. They showed that during acute illness diarrheal fluid arose throughout the small bowel, the rate of production being greatest in the proximal portion and least distally. Total small-bowel fluid production exceeded the rate of stool production, indicating that colonic absorption persisted despite its being bathed with the vibrio and its products. Studies in dogs have yielded similar results. Sack et al. (80) have shown that there is essentially no fluid production proximal to the pyloric valve or distal to the ileocecal valve of dogs with cholera induced by orogastric challenge with living *V. cholerae*. Carpenter et al. (14) have shown that challenge of canine small bowel with crude cholera enterotoxin produces the greatest rate of fluid outpouring per unit length from the duodenum and the least from the ileum. Other characteristics of the small intestinal response to cholera enterotoxin have also been studied in animal models of the disease. Carpenter et al. (14), utilizing a canine model, have shown that a single application of crude cholera enterotoxin to a Thiry-Vella jejunal loop causes fluid loss into the loop of 14 to 18 hr duration. The onset of cholera enterotoxin effect upon net absorption or secretion begins shortly after enterotoxin application but does not reach a peak until 3 to 4 hr and is sustained at near maximal levels for 4 to 5 hr, thereafter declining slowly over the next 12 hr. The fluid produced is essentially isotonic with plasma and contains less than 250 mg of protein per 100 ml (14). The composition of the fluid entering the intestinal lumen differs from plasma, having a bicarbonate content lower than plasma in the duodenum and threefold higher than plasma in the ileum. Similar differences have been observed in human cholera (5). Although the stomach does not contribute to diarrheal fluid production in cholera, its function is markedly altered during the disease, possibly by the effect of cholera enterotoxin. Studies of human cholera have shown that total gastric secretion and gastric acid production are both markedly inhibited during cholera (78). The effect of cholera enterotoxin upon the colon has not been fully evaluated.

Many early studies suggested that cholera produced extensive sloughing of intestinal mucosa (22, 59, 93) which led to massive fluid leakage from exposed submucosal vessels, though this concept was challenged as early as 1882 by Cohnheim (15). Recent studies by Gangarosa et al. (39), using a peroral biopsy technique, have shown that the intestinal mucosa is intact in cholera, and studies by Saha and Das (81) and by Gordon (43) have demonstrated that there is no increase in "leakage" of plasma protein into the bowel as might be expected to occur if mucosal denudation played a significant role in the pathogenesis of cholera. The concept that cholera alters intestinal water and electrolyte transport without structural damage to the gut mucosa was confirmed by Elliott et al. (26) who performed careful light and electron microscopic studies of the morphology of intestinal mucosa in experimental canine cholera. Their studies showed only slight capillary dilitation, lamina propria edema in the villus tips, increased production and discharge of mucus from goblet cells, and cryptal dilitation.

Despite its marked effect upon net water and electrolyte transport in the small bowel, several

other functions of the intestinal mucosa appear to be unaltered by cholera enterotoxin. Active glucose absorption and the glucose-related enhancement of sodium and water absorption are unaltered by cholera enterotoxin in man (52, 70) and in experimental animals (14, 54, 62). Similarly, the absorption of at least one actively transported amino acid, glycine, appears to be intact in human cholera (68). Keusch et al. (57) showed that the membrane-bound enzyme adenosine triphosphatase, which is magnesium dependent and activated by sodium and potassium, is also unaltered during the first 20 min after the application of cholera enterotoxin to infant rabbit jejunal mucosa. Studies of this adenosine triphosphatase activity in human cholera show it to be moderately reduced during the disease (52). Similarly depressed adenosine triphosphatase activity was noted, however, in patients with noncholera diarrhea; this could be a result of the diarrheal process rather than a direct effect of cholera toxin upon the intestinal mucosa. The studies of Keusch et al. (57) also showed that cholera enterotoxin induces no change in oxygen consumption by viable rabbit jejunal slices, when incubated together for 3 hr, or in oxidative phosphorylation by mitochondrial fractions from rabbit liver when these are incubated for 30 min. Since the onset of cholera enterotoxin effect is characteristically slow, their studies do not entirely rule out an effect occurring beyond the period of observation.

Site of Action of Cholera Enterotoxin

Present evidence strongly suggests that cholera enterotoxin produces its effect upon intestinal water and electrolyte transport by direct action upon the luminal surface of gut mucosal cells. The exact site of enterotoxin binding to, or entry into, the mucosal cell is not yet known. It is, however, clear that the enterotoxin interacts rapidly with the mucosal cell. Carpenter and Sack (*unpublished data*) have shown that cholera enterotoxin placed within a canine jejunal loop produces its full effect even if attempts are made to flush it out of the loop or to neutralize it with antitoxic serum within 1 min after its introduction. A specific interaction of cholera enterotoxin with the luminal surface of the mucosal cell is suggested by studies showing that it has no effect when applied to the serosal surface of stripped viable rabbit ileal mucosa in a dose which induces characteristic changes in ion transport and short-circuit current when applied to the mucosal surface (M. Field, D. Fromm, C. K. Wallace, and W. B. Greenough, *unpublished data*). The probability that cholera enterotoxin effect is intimately related to its binding to, or passage through, the cell membrane is suggested by studies (58, 86), discussed in greater detail below, which indicate that the cholera enterotoxin effect is mediated by its activation of mucosal cell adenyl cyclase, an enzyme which is characteristically membrane bound.

Some authors have suggested that cholera enterotoxin is absorbed and is blood or lymph borne to distal receptor sites or that it induces mucosal cell formation of a secondary circulating "messenger" substance which in turn acts upon distal receptor sites. Serebro et al. (85) reported that introduction of crude cholera enterotoxin into a carefully ligated segment of rabbit ileum induces isotonic fluid loss in that segment but that it is also accompanied by a significant reduction in isotonic fluid absorption by a second ileal segment which had not had luminal enterotoxin exposure. Although the major enterotoxin effect is clearly upon the loop to which enterotoxin was applied, their study suggests the possibility that cholera enterotoxin might also be absorbed and distributed to distal receptor sites or that it induces release of a circulating diarrheagenic "messenger" substance. These possibilities are also suggested by a series of studies on infant rabbits by Vaughan-Williams and his associates (90–92). They report that *V. cholerae* infection of a surgically constructed isolated segment of jejunum induces lethal diarrhea in infant rabbits. Furthermore, they demonstrated by cross-circulation technique that cholera infection of one infant rabbit is accompanied by an increase in intestinal water content of its noninfected partner. Although these studies do not differentiate between the possibilities that the enterotoxin is absorbed or that it induces the formation of a diarrheagenic "messenger" substance, other studies suggest that the former is unlikely. Evidence that cholera enterotoxin is poorly absorbed is based primarily upon the failure to detect circulating enterotoxin during cholera and upon the demonstration that intraluminal exposure to enterotoxin is a poor means of stimulating antitoxin production. Dogs with experimentally induced cholera have no demonstrable enterotoxin in their thoracic duct lymph (R. B. Sack and C. C. J. Carpenter, Jr., *unpublished data*). Although parenterally administered cholera enterotoxin induces high serum antitoxin titers, Curlin et al. (19) have shown that repeated challenge of a canine Thiry-Vella jejunal loop with crude enterotoxin yields no rise in circulating antitoxin titer and no decrease in loop responsiveness to subsequent enterotoxin challenges. This suggests that antigenically significant quantities of the enterotoxin molecule do not reach antibody-producing cells. This observation is supported by

human studies showing that convalescent cholera patients develop only low levels of circulating antitoxin and that this response is even poorer if antibiotics are utilized to shorten the duration of intestinal exposure to the enterotoxin (71).

Effects of Cholera Enterotoxin on Intestinal Water and Electrolyte Transport

The fluid which enters the small bowel after luminal exposure to cholera enterotoxin must be derived from blood plasma. Furthermore, it must enter the bowel as a result of alteration in either the membrane characteristics which control the flow of fluid in response to physical driving forces such as hydrostatic or osmotic pressures (i.e., permeability) or the ion-transport mechanisms which require energy expenditure, or both.

Effects on active ion transport. Huber and Phillips (53) and Fuhrman and Fuhrman (38) first suggested that cholera enterotoxin might produce intestinal fluid accumulation by inhibiting the active mucosal mechanism responsible for transport of sodium from intestinal lumen to plasma. The substance which produced this effect in their studies was, however, heat stable and dialyzable and clearly different from the toxin, subsequently purified, which is responsible for the production of diarrhea. Their studies, nevertheless, served to stimulate other studies which have attempted to define the effect of cholera enterotoxin upon active ion transport.

Studies of the effect of cholera or cholera enterotoxin on unidirectional sodium fluxes across small-bowel mucosa have been conducted in humans (6, 63), dogs (54), and rabbits (62) in attempts to better define the ion-transport alterations responsible for diarrhea. Unfortunately, consistent results have not been obtained. Several studies (6, 54, 62) suggest that the unidirectional flux of sodium from plasma to gut lumen is increased, whereas the flux from lumen to plasma is unchanged. However, studies in humans by Love et al. (63) conclude that both unidirectional sodium fluxes are decreased during cholera, the decrease in lumen to plasma flux being the greatest.

The transmural electrical potential of the intestine has been measured indirectly in humans with cholera and reported to be normal (77). However, directly measured transmural potential difference in rabbits is significantly altered by cholera enterotoxin, becoming increasingly negative and the rate of change bearing a linear relationship to the rate of fluid output (29, 66). Measurement of the active components of unidirectional ion fluxes across intestinal mucosa, however, are not possible in the intact animal. These measurements can be made when isolated, viable intestinal mucosa is stripped of its muscularis and mounted in Ussing chambers (47). In this system the measurement of short-circuit current across the membrane and the determination of unidirectional fluxes by isotopic technique permit direct determination of the active components of ion movement. Employing this technique, Field et al. (29) showed that after application of a cell-free culture filtrate of *V. cholerae* to rabbit ileal mucosa, the normal electrogenic transport of sodium from mucosa to serosa is eliminated and active chloride transport, which is normally from mucosa to serosa is reversed so that active chloride secretion occurs. Al Awqati et al. (1) utilized the same technique to carry these observations further. Studying the effect of highly purified cholera enterotoxin on normal, viable, human ileal mucosa, they have obtained the same results. Both of these effects, the inhibition of active sodium absorption and the stimulation of active chloride secretion, if occurring in vivo, would result in net water and electrolyte transport into the intestinal lumen as occurs in cholera. The demonstration that this effect is produced by purified cholera enterotoxin acting on the mucosal surface of the human ileum makes it very likely that similar changes in active ion transport do occur in vivo.

Effects on passive permeability. Alterations in permeability which might lead to an increased flow of water and solute from blood plasma to intestinal lumen can be thought of as affecting the entire series of membranes between capillary and intestinal lumen or reducing the impedance to flow through real or potential extracellular passages of the gut mucosa, or both. In any case, increased flow could result only if there existed a significant hydrostatic or osmotic driving force to effect fluid movement. The demonstration by Craig (17) that cholera enterotoxins, both crude and highled purified, cause a marked increase in capillary permeability to serum protein when injected intracutaneously would appear to support the possibility that cholera enterotoxin might alter permeability in the mucosal capillaries of the intestine. However, there is no evidence to indicate that this does occur. Cholera in humans and experimental animals is not associated with increased protein movement into the thoracic duct lymph (13) or into the bowel lumen (43, 72). Neither is it accompanied by any electron microscopic evidence of loss of capillary or mucosal membrane integrity or interruption of the tight junction between adjacent mucosal cells (26).

These observations do not exclude the possibility that permeability to much smaller molecules (i.e., sodium, chloride, etc.) might be increased in

cholera. Even so, there is still the requirement that such an increase in permeability be coupled with an osmotic or hydrostatic driving force to produce increased transmucosal flow. Available evidence indicates little, if any, difference in osmotic activity of blood and small-bowel fluid induced by cholera enterotoxin (14), suggesting that a significant osmotic gradient is not present. It remains possible that mucosal capillary hydrostatic pressure plays a role in fluid movement. Unfortunately, there is not yet sufficient data to fully assess this possibility. If capillary hydrostatic pressure plays a major role in the fluid production induced by cholera enterotoxin, one would predict that variations in transmucosal pressure gradients would greatly influence the rate of fluid loss. Studies pertinent to this possibility are described below.

Role of mesenteric blood flow. If an increase in permeability between the vascular bed and the intestinal lumen contributes to fluid loss in cholera, it might be expected that the rate of fluid loss would be related to the magnitude of intestinal blood pressure and flow. Utilizing the canine model, Carpenter et al. (13) showed that superior mesenteric artery flow rates are normal during intestinal fluid loss induced by luminal application of cholera enterotoxin if the dog is maintained in normal hydration. If dehydration occurs, diarrhea continues at a constant level despite a marked reduction in mesenteric blood flow. Furthermore, reduction of mean superior mesenteric artery pressure by means of a snare to levels below 30% of normal produced no decrease in the rate of enterotoxin-induced fluid output by the small bowel. Their data suggest that a blood pressure-dependent passive movement of fluid from blood to intestinal lumen is not an important effect of cholera enterotoxin. The possibility that the distribution of blood flow within the intestinal mucosa is altered in response to cholera enterotoxin has not been carefully studied. This possibility is suggested, however, by studies of experimental canine cholera by Elliott et al. (26) which show that capillaries of the villus tip are distended, whereas those in the crypt region of the mucosa are somewhat diminished in size. Despite these changes, it seems unlikely that changes in the mucosal microcirculation could be sufficient to compensate for the wide range of mesenteric artery pressures employed in the studies cited above.

Effects of Cholera Enterotoxin on Nonintestinal Tissues

Recent studies have shown that cholera enterotoxin, even in its purified form, affects the function of a wide variety of tissues. Although these effects contribute little, if any, to the disease state seen in human cholera, their study is of importance in understanding the cellular mechanism of action of cholera enterotoxin and in providing isolated cell systems in which to study this effect.

Increased skin capillary permeability. Basu Mallik and Ganguli (7) first noted that cholera stool filtrates injected intracutaneously in rabbits caused an increase in skin capillary permeability to protein. Craig has shown that this effect is also produced in rabbits and guinea pigs by cell-free filtrates of *V. cholerae* culture and that the effect is neutralized by convalescent sera from cholera patients (17). Despite reports (44, 61) that fractionation of crude cholera enterotoxin permits separation of the skin vascular permeability factor and the diarrhea-producing factor, several lines of evidence strongly suggest that altered skin capillary permeability is produced by the same enterotoxin responsible for diarrhea. To date the most highly purified forms of cholera enterotoxin are highly active in producing increased skin capillary permeability (30, 74). The capacities of sera from convalescent patients to neutralize the skin capillary permeability changes and the diarrheagenic potential of cholera enterotoxin rise and fall precisely in parallel (67, 71). This would be unlikely if these neutralizing capacities were stimulated by different antigens.

Production of edema in rat footpad. Injection of cholera enterotoxin into the foot of a rat produces, after a 2- to 4-hr delay, a prolonged but reversible local edema, the severity of which is dose dependent (33). Similar changes also occur in the mouse (32). The cholera enterotoxin-induced edema lasts for 5 or more days, depending upon toxin dose.

Enhancement of lipolysis by rat epididymal fat cells. Vaughan et al. (89) showed that incubation of isolated viable rat epididymal fat cells with either crude or purified cholera enterotoxin results, after a delay of 2 hr, in an increased rate of lipolysis by these cells as indicated by an increase in the rate of release of glycerol into the incubation medium. Enterotoxin boiled 5 min prior to incubation produced no effect upon lipolysis. The stimulation of lipolysis has been shown to be proportional to the log of enterotoxin concentration and is neutralized by cholera antitoxin, thus permitting this system to be easily adapted to the measurement of enterotoxin and antitoxin activity (48).

Enhancement of glycogenolysis in platelets and liver. Graybill et al. (45) showed that purified cholera enterotoxin produces hyperglycemia of at least 48 hr duration after intravenous injection

of as little as 10 μg in a dog. The effect is not produced by preboiled enterotoxin. Further studies of this effect by Zieve et al. (94) have shown that purified cholera enterotoxin markedly enhances the rate of glycogenolysis in liver after intravenous injection in mice. Similar increases in rates of glycogenolysis have been observed by the same authors in sonically treated human platelets and homogenized rat liver after incubation with enterotoxin in vitro. These studies indicate that cholera enterotoxin enhances glycogenolysis by increasing the activity of phosphorylase *a* within these cells. Their studies also demonstrate an almost immediate onset of enterotoxin effect upon disrupted cells. This observation is in marked contrast to the delay of 60 to 120 min before the onset of enterotoxin effect in all other systems studied including fluid production by the intact small bowel (14), lipolysis in isolated rat epididymal fat cells (89), capillary permeability in guinea pig skin (17), and hyperglycemia and hyperalkaline phosphatasemia in dogs (45). This suggests that the commonly observed delay in onset of enterotoxin effect may be due to delay in its entry into the intact target cell, the delay being eliminated by mechanical disruption of the cell.

Enhancement of hepatic alkaline phosphatase production. Graybill et al. (45) also showed that intravenous injection of as little as 10 μg of purified enterotoxin into dogs induces elevation of serum levels of alkaline phosphatase of hepatic origin which last at least 48 hr. This effect is accompanied by little evidence of hepatic cell damage by the enterotoxin and further studies (N. F. Pierce, J. R. Graybill, M. M. Kaplan, and D. Bouwman, *unpublished data*) have shown that serum alkaline phosphatase rises as a result of increased alkaline phosphatase synthesis by hepatic cells. This effect is not produced by preboiled toxin.

Alteration of Cholera Enterotoxin Effects by Pharmacological Agents

Two drugs, ethacrynic acid and cycloheximide, have been shown to reverse some of the effects of cholera enterotoxin. Although neither is suitable as a therapeutic agent for use in humans, their effects suggest that other pharmacological agents might be found which would reverse cholera enterotoxin effects and be safe for human use. These agents also serve as useful tools for better understanding of the mechanism of action of cholera enterotoxin.

Effect of ethacrynic acid upon cholera enterotoxin effects. Carpenter et al. (12) showed that intravenous or intraluminal ethacrynic acid will significantly decrease the rate of intestinal fluid loss after intestinal challenge with cholera enterotoxin. The effect of ethacrynic acid upon the rate of fluid production is not apparent until 2 to 3 hr after its administration and the effect lasts for at least 7 hr. Similarly, Al Awqati et al. (3) showed that ethacrynic acid largely inhibits the cholera enterotoxin-induced increase in short-circuit current across isolated, stripped, viable rabbit ileal mucosa. They suggest that ethacrynic acid acts in some way to inhibit the anion-secreting process which cholera enterotoxin activates.

Ethacrynic acid also inhibits the effect of cholera enterotoxin in at least one system in which altered electrolyte transport may not be a significant component of the enterotoxin effect. Vaughan et al. (89) clearly showed that ethacrynic acid completely inhibits the effect of cholera enterotoxin upon glycerol release by rat epididymal fat cells.

Effect of cycloheximide upon cholera enterotoxin effects. Serebro et al. (84) reported that cycloheximide, an inhibitor of protein synthesis, will prevent fluid production in rabbit intestinal loops if administered intravenously 1 hr before placing enterotoxin in the intestinal loop. In further studies, Harper et al. (50) and Grayer et al. (46) showed that cycloheximide administered up to 2 hr after enterotoxin challenge markedly diminished the rate of fluid production during the next several hours. They also showed that cycloheximide administration led to increased rates of net absorption in control loops of intestine. In both instances, cycloheximide administration was followed by a reduction in the sodium flux from plasma to gut lumen but produced no change in sodium flux from lumen to plasma. Active absorption of glucose from the intestinal lumen was not altered by cycloheximide, but cycloheximide did produce mitotic arrest and degenerative changes in crypt cells. The authors speculate that cycloheximide interrupts a protein synthetic step, probably in the mucosal crypts, which is necessary to establish and maintain enterotoxin-induced fluid output.

Cycloheximide has also been shown by Finkelstein et al. (33) to prevent or reverse the local edema which follows injection of cholera enterotoxin into the rat footpad. Intravenous injection of cycloheximide up to 2 hr after cholera enterotoxin injection delayed the onset of cholera enterotoxin effect by about 8 hr. Cycloheximide injections at 0 and 10 hr after cholera enterotoxin further delayed the onset of edema until 36 hr after injection. The fact that edema then occurred clearly demonstrates the long duration of cholera enterotoxin effect upon exposed tissues.

Role of Cyclic 3'5' Adenosine Monophosphate (cAMP) in Mechanism of Action of Cholera Enterotoxin

Several recent studies strongly suggest that cholera enterotoxin produces its effects, in both intestinal and nonintestinal tissues, by altering tissue levels of cAMP. This conclusion has been supported by studying the effect upon intestinal fluid transport of pharmacological agents capable of altering tissue cAMP levels (i.e., prostaglandins and theophylline) and by direct measurement of the effect of cholera enterotoxin on adenyl cyclase and cAMP levels in intestinal tissue. These studies provide the most convincing evidence yet available of a specific mechanism of action of cholera enterotoxin.

Effects of prostaglandins, theophylline, and cAMP on intestinal water and electrolyte movement and their relation to the effects of cholera enterotoxin. Field et al. (30), using the short-circuited preparation of rabbit ileal mucosa described above, first showed that theophylline and dibutyryl cAMP each produce a marked increase in short-circuit current which is associated with inhibition of active sodium absorption and stimulation of active chloride secretion. This observation suggested that an increase in mucosal cell cAMP, produced either by addition of cAMP to the bathing solution or by theophylline inhibition of the phosphodiesterase which breaks down intracellular cAMP, resulted in changes in ion transport which could result in vivo in the accumulation of fluid within the intestinal lumen. In a subsequent study, Field et al. (29) showed that crude cholera enterotoxin produced a similar rise in short-circuit current and also inhibited sodium absorption and stimulated chloride secretion by rabbit ileal mucosa. Furthermore, they showed that the theophylline effect upon short-circuit current, described above, was significantly reduced in tissues pretreated with cholera enterotoxin, suggesting that these agents act upon the same secretory mechanism. This observation provided the basis for several further studies which have established the biochemical mechanism of action of cholera enterotoxin upon mucosal cell ion transport. In further studies, Al Awqati et al. (1) showed that purified cholera enterotoxin produces the same changes when applied to stripped, viable, human ileal mucosa. Another agent, prostaglandin E_1, which alters cAMP levels in a variety of tissues by altering adenyl cyclase activity, has also been studied in the same system. Al Awqati et al. (2) showed that prostaglandin E_1 also produces the same changes in sodium and chloride movement as observed with cAMP, theophylline, and cholera enterotoxin.

These in vitro studies have been extended to an in vivo system to determine their validity in the intact animal. Pierce et al. (72) showed that theophylline and several prostaglandins (PGE_1, PGA_1, and $PGF_{2\alpha}$) are capable of inducing loss of water and electrolytes from canine small bowel when infused into the mesenteric artery. The intestinal fluid produced was similar in electrolyte content to that induced by cholera enterotoxin. The demonstration that theophylline and prostaglandin $F_{2\alpha}$ acted synergistically in inducing intestinal fluid loss suggested that these agents acted upon different components of a single secretory mechanism. Furthermore, there was a significant correlation between the magnitude of effect of intraarterial prostaglandin and of intraluminal cholera enterotoxin when studied in the same dog, again suggesting that these agents stimulate the same small bowel secretory mechanism.

The strong suggestion that cholera enterotoxin exerts its effect upon intestinal ion transport by activating a cAMP-mediated system has now been confirmed. Kimberg et al. (58) and Sharpe et al. (86) have shown that cholera enterotoxin markedly enhances gut mucosal cell adenyl cyclase activity while having no effect upon phosphodiesterase activity. The time course of increase in adenyl cyclase activity is similar to the time course of fluid output in response to the enterotoxin, rising slowly to a peak at about 3 hr. Kimberg et al. (58) also showed that prostaglandin E_1 and other prostaglandins markedly enhance mucosal cell adenyl cyclase activity. Finally, Kimberg et al. (58) and Schafer et al. (83) showed that cholera enterotoxin induces a marked increase in mucosal cell content of cAMP.

This demonstration that a bacterial product produces its effect by alteration of a cAMP-dependent cellular function is not new. Macchia et al. (64) previously showed that *Clostridium perfringens* produces a factor which stimulates thyroid hormone production, another cAMP-mediated process.

Relation of cAMP to extraintestinal effects of cholera enterotoxin. It is likely that several, and perhaps all, of the extraintestinal effects of cholera enterotoxin are also mediated by an effect upon cellular cAMP levels.

The rate of lipolysis by rat epididymal fat cells is known to be controlled by cellular cAMP levels, being enhanced by hormonal agents which raise cAMP levels. Enhancement of fat-cell lipolysis by cholera enterotoxin (89) certainly suggests that the enterotoxin may also act by

increasing cellular cAMP levels. The inhibition of the enterotoxin-induced increase in lipolysis by ethacrynic acid (89) is a second example of ethacrynic acid antagonism of cholera enterotoxin effect, and it raises the possibility that ethacrynic acid might act by reversing an enterotoxin-induced increase in adenyl cyclase activity.

The enhancement of glycogenolysis in platelets and liver by cholera enterotoxin also suggests an effect of the enterotoxin on cAMP activity. In these cells cAMP accelerates the activation of phosphorylase kinases which in turn facilitate the conversion of phosphorylase *b* to *a*. Phosphorylase *a* accelerates glycogen catabolism, leading ultimately to glucose release. The demonstration that cholera enterotoxin has no direct catabolic effect upon free glycogen but does enhance glycogenolysis in broken cells in association with elevated levels of phosphorylase *a* again suggests that the enterotoxin may act by a mechanism which increases cAMP activity (94).

The effects of cholera enterotoxin upon skin vascular permeability, rat footpad edema, and hepatic alkaline phosphatase production are not sufficiently well understood to permit speculation as to whether enhanced cAMP activity might also play a role in their genesis.

Duration of cholera enterotoxin-induced effects. A striking characteristic of every effect of cholera enterotoxin observed to date is its prolonged duration after brief enterotoxin exposure, with ultimate return to normal function. The duration of effect upon mucosal water and electrolyte transport in the intestine does not exceed 36 hr and is possibly limited by the complete replacement of mucosal cells during this period. Other effects [i.e., duration of hyperglycemia and hyperalkaline phosphatemia in dogs (N. D. Pierce, J. R. Graybill, M. M. Kaplan and D. Bouwman, *unpublished data*) and edema in the rat foot (33)] exceed 5 days. If cholera enterotoxin achieves this effect by alteration of cellular cAMP levels, it stands in contrast to virtually all other agents which have been shown to do so, the usual pattern being a rapid onset of effect after exposure of the tissue and a rapid return to normal activity when the stimulating agent is removed. This characteristic of cholera enterotoxin and the delay in onset of its effect upon intact cells may be major clues to its mechanism of action.

DIARRHEAGENIC TOXINS FROM OTHER ENTERIC ORGANISMS

There is growing evidence that other enteric organisms are capable of producing diarrheagenic enterotoxins which may play a role in the production of diarrhea by these agents. Some reports suggest that some of these enterotoxins may be similar to cholera enterotoxin. To date, enterotoxic activity has been demonstrated in cell-free culture supernatant fluid or cell lysates of *Escherichia coli* (49, 65, 87), *C. perfringens* (23), and *Shigella dysenteriae* (56).

Certain strains of *E. coli* are enteropathogenic for swine, being similar to cholera in severity and in that the causative organisms reside only within the intestinal lumen. Several reports (49, 65, 87) have described the presence of diarrheagenic enterotoxin(s) in cell-free culture supernatant fluids of these organisms or in their cell lysates. Both heat-stable and heat-labile enterotoxins have been described (49, 65, 87). A causative role of these enterotoxins in diarrhea production is suggested by the observation that nonenteropathogenic strains of *E. coli* cannot be shown to produce enterotoxin(s) in vitro. Heat-labile *E. coli* enterotoxin has been shown to be nondialyzable, to be precipitated by ammonium sulfate, and to withstand lyophilization (49, 65). Furthermore, the heat-labile enterotoxin causes fluid accumulation in both pig (49) and rabbit (65) intestinal loops, and this effect is neutralized by prior incubation with antisera against the whole living cell, its dialyzed enterotoxin, and against *V. cholerae* enterotoxin (49). Finally, a further suggestion of a relationship between this *E. coli* enterotoxin and cholera enterotoxin lies in the observation that a crude *V. cholerae* enterotoxin is neutralized by antiserum against living enterotoxigenic *E. coli* (49).

Several reports suggest that *E. coli*, which do not belong to the group of enteropathogenic *E. coli* (E.E.C.) serotypes commonly associated with nursery outbreaks, may cause diarrhea in humans. Such organisms could account for a portion of the 60 to 80% of acute diarrheal disease in which recognized pathogens are not isolated from stool. Sakazaki et al. (82) reported that certain *E. coli* serotypes which are not among the common infantile E.E.C. serotypes are commonly found in Japan among children and adults with diarrhea. Rowe et al. (76) described an outbreak of diarrhea associated with a single unusual *E. coli* serotype. Finally, Banwell et al. (4) and Gorbach et al. (41), studying acute undifferentiated diarrhea among adults in Calcutta, showed that half of the patients studied yielded large numbers of *E. coli* in pure growth from their upper jejunum during acute disease. Each patient yielded a single serotype; however, the serotypes differed from patient to patient. Fluid loss into the jejunum was demonstrated in most of these patients. Finally, the *E. coli* strains isolated from these persons have been shown to produce an enterotoxin in the cell-

free culture supernatant fluid which causes fluid accumulation in ligated rabbit ileum (40) and outpouring of fluid from the canine jejunum (N. F. Pierce and C. K. Wallace, *unpublished data*).

An enterotoxin capable of causing diarrhea in rabbits and fluid accumulation in the rabbit ileal loop has also been demonstrated in cell lysates and culture supernatant fluids of strains of *C. perfringens* associated with human food poisoning. The enterotoxic activity described also has some similarities to that of cholera enterotoxin, being heat labile, nondialyzable, Pronase sensitive, typsin resistant, and acid labile. Its effect upon rabbit ileal loops was also characterized by a lag of 3 hr before the onset of demonstrable fluid accumulation (23).

Finally, Keusch et al. (56) reported the isolation of a diarrheagenic enterotoxin present in cell-free culture supernatant fluids of *Shigella dysenteriae*. This enterotoxin is heat labile, distinct from endotoxin and neurotoxin, has an apparent MW of about 50,000, and produces fluid accumulation in rabbit ileal loops in submicrogram amounts. The authors suggest that the diarrhea accompanying infection with this strain may be mediated by this enterotoxin.

These observations suggest that various organisms are capable of producing enterotoxins which cause diarrhea arising in the small bowel. Of particular interest is the observation that some of these organisms are normal members of the fecal flora. The factors which permit these organisms to invade the small bowel, at least temporarily, are understood very poorly. The possibility that other organisms normally found in feces may also produce similar enterotoxins certainly bears investigation. It is possible that *E. coli*, and perhaps other normal members of the fecal flora, may be causative of a large portion of acute diarrheal disease from which no recognized enteric pathogens are recovered.

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